Adenovirus Infection Dramatically Augments Lipopolysaccharide-Induced TNF Production and Sensitizes to Lethal Shock[1]

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We observed a remarkable synergism of adenoviruses and LPS in triggering the production of TNF in intact animals. We found that in mice pre-exposed to adenoviruses, LPS injections generated extremely high levels of TNF with altered kinetics. The elevated TNF synthesis stemmed mostly from posttranscriptional up-regulation of TNF production, although transcription of the TNF gene was also induced. Adenoviruses and LPS exhibited a significant but less dramatic synergism in the induction of IL-6, IFN-γ, and NO. Only marginal changes were detected in the synthesis of a panel of other cytokines. Different serotypes of the virus showed practically identical effects. As deletion mutants lacking indispensable viral genes or UV inactivated virions exhibited similar activities as the infectious, wild-type virus, it seems unlikely that the viral genome plays any significant role in the phenomenon. Published data indicate that other viruses also show some kind of synergism with LPS, although by different cellular mechanisms. T cells and their IFN-γ production—crucial in the synergism of influenza viruses and LPS—were dispensable in our experiments. We suggest that the phenomenon is probably a general one: an overlap between different molecular mechanisms detecting bacterial and viral pathogens and inducing mediators of nonspecific cell-mediated host defense. The synergism of viruses and LPS (bacteria) could be a concern in medical practice as well as in gene therapy experiments with high doses of recombinant adenoviruses. ***The Journal of Immunology,*** **2005, 175: 1498–1506.**

Millions of years of co-evolution resulted in an intricate interrelationship between pathogenic organisms and the immune system of their hosts. During the last decade, a number of publications reported "intelligent" viral mechanisms—mostly by large DNA viruses—that interfere with the complement system, prevent inflammation, or block Ag presentation and activity of immune cells (1–3). Similar strategies are described as virulence factors in many pathogenic organisms, from bacteria to nematodes (3, 4).

Among other inflammatory cytokines, TNF plays important roles in controlling virus infections directly by inducing apoptosis or necrosis of virus-infected cells and indirectly by mobilizing and activating immune and vascular endothelial cells (5). Nevertheless, uncontrolled production of TNF can be harmful to the organism. High levels of systemic TNF cause very severe side effects (like high fever, septic shock-like syndrome, or disseminated intravascular coagulation), and even low concentrations are detrimental if present for long periods of time (chronic inflammation, wasting, cachexia) (6).

Therefore, the production of TNF is under very strict regulation involving levels of gene activity, mRNA stability, and translation (7, 8). Shedding of the paracrine form of the cytokine and/or its receptors is also regulated (9). In inflammatory reactions the major source of TNF are macrophages and activated leukocytes (5). The production of TNF and other proinflammatory cytokines are triggered by various products of bacteria including endotoxin, lipoproteins, superantigens, or bacterial DNA (5, 10). Among these inducers LPS (endotoxin), a bacterial cell wall component is one of the most potent alarm signals in innate immunity and one of the best inducers of TNF. Up-regulation of TNF production by endotoxin is the result of increased steady-state levels and enhanced translation of the TNF mRNA (10).

Adenoviruses have a complex relationship with TNF. This cytokine triggers signaling pathways leading to apoptosis of cells expressing the adenoviral *E1A* gene and inhibits assembly and maturation of adenoviral particles (11, 12). In contrast, the adenovirus *E1B* 19K protein inhibits TNF-induced apoptosis and several transcripts of the *E3* gene (10.4/14.5 K, 14.7 K), induced by TNF itself, protect infected cells against TNF cytotoxicity by different molecular mechanisms (13, 14).

We have shown previously that the expression of the *E3* gene is activated by LPS in various tissues of *E3* transgenic animals (15). Because adenoviruses and endotoxin may occur simultaneously in different situations, we studied the combined effect of these agents on innate immune responses. The data presented here show that adenovirus infection augments the production of certain LPS-activated mediators, especially TNF. Possible mechanisms and implications for pathogenesis of this intriguing synergism between different activators will be discussed.

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Received for publication June 8, 2004. Accepted for publication May 10, 2005.

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[1] This work was supported in part by the Hungarian National Science Foundation (OTKA T029458) (to T.P.) and (OTKA D25321) and Bolyai J. Research Fellowship (to G.F.) and from the Hungarian Ministry of Health (FKFP 0659/99) to (G.L., Sr.).

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0022-1767/05/$02.00

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Materials and Methods

Viruses

R700 is a replication-competent Ad5 having a partially recombined *E3* region from Ad2; these viruses were grown in Hep2C suspension cultures. Wild-type Ad3 and Ad12 viruses and dl312, a replication defective Ad5 that does not express E1A proteins, were grown in 293 cells. All the viruses used were freon extracted and purified by CsCl equilibrium gradient centrifugation as described earlier. In the case of R700 and Ad3, an additional band of empty capsids (above the mature virions) was also harvested. This was verified as empty capsids based on the presence of viral proteins on SDS-PAGE and the absence of infectivity and unit length viral DNA, and density after the second round of CsCl gradient centrifugation.

All virus preparations were dialyzed against PBS containing 15% glycerol and stored in aliquots at −80°C. R700 was plaque titrated on both A549 and 293 cells, dl312, Ad3, and Ad12 on 293 cells. UV-inactivated viruses were obtained by irradiating virus samples on ice with continuous shaking as described earlier (16). Heat inactivation was done by incubating virus preparations at 65°C for 1 h. Proteinase K inactivation was done by treating the virus sample with 200 μg/ml enzyme at 37°C for 1 h. These procedures lowered viral infectivity by >100,000-fold, as assessed by plaque assay. The absence of endotoxin was demonstrated in the different viral preparations with a Limulus assay.

Mice

Throughout these studies, 8- to 10-wk-old animals were used. C57BL/6 male specific pathogen-free mice, vaf+ C57BL/6 male mice, and CD1 nu/nu male vaf+ mice were purchased from Charles River Hungary. IFN-γ −/− C57BL/6 male vaf+ mice were purchased from The Jackson Laboratory.

Adenoviruses were administered to the animals i.p. or i.v., in amounts specified for each experiment. *Escherichia coli* LPS (serotype O55:B5 obtained from Sigma-Aldrich) was injected in 0.1 ml of PBS per animal— the amount and route (i.p. or i.v.) indicated at each experiment. Control animals were treated with PBS alone.

For the lethality experiments, mice were injected i.p. with the virus doses indicated; 16 h later, LPS was given i.p. at specified doses. Animals were kept in individual cages, body temperature values were monitored with an electronic thermometer, and survival was recorded.

In vitro LPS stimulation of human PBMC pretreated with inactivated adenovirus

PBMC freshly isolated from human buffy coat (Transfusion Center) by Ficoll-Paque Plus (Amersham Biosciences) centrifugation were suspended in RPMI 1640 medium, supplemented with 25 mM HEPES buffer, 300 mg/l L-glutamine, 1% FBS (all obtained from Invitrogen Life Technologies), 50 μg/ml gentamicin (Sigma-Aldrich) and plated on 96-well plates (MTP, Falcon; BD Biosciences) at a cell density of 5×10^5 cells/well. Cells were treated with a 10-fold or 100-fold number of UV-inactivated adenovirus particles in a total volume of 200 μl/well for 16 h at 37°C, 5% CO_2. After incubation, 20 μl of 5000 pg/ml LPS (*E. coli* O26:B6 serotype; Sigma-Aldrich) was added to each well (except to the appropriate controls), and plates were incubated for an additional 16 h (same conditions). Some wells received different amounts of TNF inhibitor, pentoxifylline (50, 100, and 200 μg/ml) (Sigma-Aldrich) 1 h before LPS stimulation. Six parallels were used on each plate. Supernatants were tested for cytotoxicity as well as for TNF concentration. Samples from wells not containing PBMC served as background.

A commercial, colorimetric LDH cytotoxicity detection kit (Boehringer Mannheim) with an ELISA reader (iEMS; Labsystems Oy) was used for testing the direct cytotoxicity of the inactivated adenovirus and the TNF inhibitors according to the manufacturer's manual.

Detection of cytokines

Soluble and transmembrane forms of murine TNF-α were detected from serum as well as from homogenized organ samples with a sensitized L929 cells bioassay (17) with a detection limit of 3 pg/ml (0.15 U/ml) TNF, and also with sandwich ELISA using an Ab pair from Genzyme (coating Ab, 1221–00; biotinylated detecting Ab, 80–4895) with a detection limit of 200 pg/ml TNF. TNF concentration of human PBMC supernatants was determined by both a commercial human TNF-α ELISA kit (Boehringer Mannheim) and bioassay using A9 fibrosarcoma cell line (American Type Culture Collection) and the WST-1 cell proliferation reagent (Boehringer Mannheim). For calculating the sample TNF concentrations by linear regression, a five-point calibration curve was used. Murine IL-6 was detected on the basis of its biological activity using the B9 cell proliferation assay with a detection limit of 40 U/ml, as described earlier (18). Murine IFN-γ and IL-10 were detected with sandwich ELISA plates from HyCult and Genzyme, respectively. All the commercial reagents were used according to the suggestions of the manufacturers.

Kupffer cell blockade

Kupffer cell blockade was induced by gadolinium chloride as published earlier (19).

Analysis of RNA

Total cellular RNA was prepared from liver, lung, kidney, and spleen with the guanidium isothiocyanate method according to standard protocols, and the integrity of the RNA samples was checked by running 5 μg of RNA on formaldehyde gels.

RNase protection assay was conducted with the ML-11 multiple probe set using 10 μg of total cellular RNA essentially as described earlier (20). This set detects transcripts of 10 different murine cytokine genes (lymphotoxin α, TNF-α, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, and IFN-γ) and the housekeeping L-32 gene. In each gel, 1/10 part of the total amount of the probe was always included as m.w. marker. TNF mRNA was also detected on Northern blots using 10 μg of total RNA for electrophoresis. For these assays, a DNA fragment containing the first 885 bp of the coding region of the murine TNF gene from the pGMTNF-MJ plasmid (a kind gift from Dr. J.-F. Arrighi) was used as probe. After stripping, blots were rehybridized with a GAPDH probe, pHcGAP (from American Type Culture Collection) for blotting control. RNase protection assay gels and Northern blots were visualized and quantified by using a PhosphorImager 445SI (Molecular Dynamics).

Real-time RT-PCR was used for the quantitative measurement of murine TLR4 RNA. Total cellular RNA was reverse transcribed with Superscript II (Invitrogen Life Technologies) using oligo(dT) priming according to the suggestions of the manufacturer. Real-time PCR was performed in a LightCycler instrument (Roche) using the primers 5′-TCATGGCACTGT TCTTCTCCTGC and 5′-GGTGTAAGCCATGCCATGCC for murine TLR4 and 5′-gtccacacccgccaccagttcg and 5′-ggaatacagcccggggagcatcgtc for murine β-actin.

Detection of NO

NO from different organs was detected using electron spin resonance basically as described earlier (21, 22). Animals were injected i.p. with the indicated amounts of viruses on day 1. LPS at the indicated dose was given i.p. 16 h later; 40 h after the virus injection, 0.5 mg/g of body weight DETC[4] was injected i.p. and 0.05 mg/g ferrous sulfate and 0.85 mmol/g sodium citrate was administered s.c. Thirty minutes after diethyldithiocarbamate (DETC)/$FeSO_4$ treatment, the animals were sacrificed, and small tissue pieces (~100 mg) from lung, liver, spleen, and kidney were immediately frozen on dry ice. Frozen tissues were then analyzed with electron spin resonance. Conventional X-band electron paramagnetic resonance spectra of NO trapped with Fe(II)DETC2 were recorded at 165 K. The partially overlapping triplet, at somewhat lower magnetic fields than the center of these spectra, is the perpendicular component of the well known signal for NO trapped by Fe-dithiocarbamate2 type of complexes, whereas the quartet signal (centered around 3285 Gauss with a splitting of ~48 Gauss) originates from DETC complexes with endogenous copper. This second signal was present in all preparations and it was therefore necessary to compare the Fe-DETC2-NO signal with a constant reference to quantify the relative amounts of trapped NO in different preparations. This was achieved by scaling a simulated triplet, to match the corresponding component in the experimental line shape. The same technique, namely electron paramagnetic resonance detection of the NO-Fe-DETC2 complex, has been used to study the effect of L-arginine inhibitors on LPS-treated mice (22).

Results

Adenovirus infection dramatically increases serum TNF levels of endotoxin-infused mice

Adenovirus R700 infection without LPS did not result in measurable TNF levels (Fig. 1). As expected, in control B6 mice treated with LPS alone, TNF production peaked 1 h after i.p. LPS infusion, dropped significantly by 2 h, and was practically undetectable at the 3 and 4 h time points.

[4] Abbreviation used in this paper: DETC, diethyldithiocarbamate.

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FIGURE 1. The influence of adenovirus infection on LPS-induced serum TNF levels. C57BL/6 (B6) mice, non-infected (solid bars) or Ad R700 infected (3×10^8 PFU per animal, empty bars) were challenged with 1 μg/g body weight LPS. Sera were taken from the groups of animals (6–8/group) at the indicated times after endotoxin treatment; 0 h represents animals that received PBS instead of LPS. Ad infection and LPS treatment were done i.p.; TNF was measured with bioassay (*A*) and with ELISA (*B*). Data are presented as the mean ± SEM.

In mice infected with Ad R700 i.p. and challenged with LPS 16 h later, serum TNF levels were drastically higher than in mice treated with LPS alone. The kinetics of TNF production was also different, maximal values were measured at 2 h after LPS exposure, and TNF was still high after 4 h.

Serum samples of these animals were analyzed for TNF with bioassay (Fig. 1*A*) and with ELISA (Fig. 1*B*), and the data obtained were very similar. The kinetics of TNF induction was practically identical in animals treated with LPS and with adenovirus (data not shown).

After the initial observations, we studied the influence of the time interval between adenovirus infection and LPS challenge on the synergism of these factors. Maximal induction of serum TNF levels were detected in animals infused with LPS 16 h after the adenovirus infection. Low level of induction was observed when LPS treatment followed the adenovirus exposure by 7 h, no augmentation occurred before 2 or after 40 h (Fig. 2).

FIGURE 2. Adenovirus elicited augmentation of LPS-induced TNF production is dependent on the time period between Ad infection and endotoxin challenge. B6 mice infected i.p. with Ad R700 (3×10^8 PFU/animal) were injected i.p. with 1 μg/g body weight LPS after the indicated time following Ad infection. Sera were obtained 2 h after endotoxin challenge. Data are presented as the mean ± SEM.

Based on these data, the majority of the subsequent experiments were conducted with i.p. adenovirus exposure and a subsequent i.p. endotoxin treatment 16 h later, measuring TNF levels 2 h after LPS challenge.

Dose-dependent synergism between adenoviruses and endotoxin

Four different doses of Ad R700 were used before the LPS challenge to test the dose dependence of the adenovirus effect on LPS-induced TNF production, as specified in the legend of Fig. 3*A*. When compared with the uninfected mice, the smallest Ad R700 dose (3×10^7 PFU/mice) did not increase significantly the LPS-triggered serum TNF level of the animals. However, infection with doses of 1×10^8 and 3×10^8 PFU resulted in >10 and 40-fold increase in TNF levels. An even larger dose of adenovirus (9×10^8 PFU) induced approximately the same values—showing a saturation effect of the adenovirus infection. In another experiment, three different LPS doses were used, and synergism was demonstrated over a 25-fold range using 3×10^8 PFU Ad R700 for infection (Fig. 3*B*).

Intact virions are necessary for the enhancement of LPS-induced TNF production, but viral gene expression is not essential

To determine whether expression of the *E1* and *E3* genes have any influence on the effect of adenovirus, B6 mice were infected with different adenovirus mutants (affecting early genes) and challenged with LPS. In mutant dl309, the anti-TNF genes of the early region 3 are deleted. In dl312, these genes and the *E1A* region are missing. Both dl309 and dl312 synergized with LPS to a similar extent as Ad R700. The results of experiments conducted with these mutant viruses are presented on Fig. 4*A*.

In the absence of the *E1A* region, expression of all other adenovirus genes are dramatically reduced. To our surprise, dl312 was as active as Ad R700. Therefore, we wanted to test whether viral

FIGURE 3. Dose dependence of the Ad-endotoxin synergism. B6 mice were uninfected or infected with Ad R700 and 16 h later treated with LPS i.p. Sera were taken 2 h after endotoxin challenge, and TNF was measured with bioassay. *A*. Animals were given 1 μg/g body weight LPS (1–5) or no LPS (6). Different amounts of Ad R700 were injected 16 h earlier: 1, no virus; 2, 3×10^7 PFU; 3, 1×10^8 PFU; 4, 3×10^8 PFU; 5, 9×10^8 PFU; and 6, 9×10^8 PFU. *B*, Animals were uninfected (solid bars) or infected with 3×10^8 PFU Ad R700 (empty bars). Different amounts of LPS were administered: 1 and 2, none; 3 and 4, 0.2 μg/g body weight; 5 and 6, 1.0 μg/g; and 7 and 8, 5 μg/g. Data are presented as the mean ± SEM.

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FIGURE 4. Intact virions are necessary to augment LPS-induced TNF synthesis. Viral gene expression is dispensable. *A*, B6 mice (5/group) were uninfected or infected with different Ad deletion mutants. *B*, B6 mice (5/group) were uninfected or infected with UV-, heat-, or proteinase K-inactivated Ad R700. All animals were challenged with 1 μg/g body weight LPS, and sera were taken at 2 h after endotoxin treatment. TNF was measured with bioassay. Data are presented as the mean ± SEM.

gene expression is necessary at all for the observed synergism. To fully diminish adenovirus gene expression, Ad R700 was UV inactivated (as described in *Materials and Methods*) and used to treat mice before LPS challenge. As shown on Fig. 4*B*, UV-inactivated Ad R700 was as effective as replication-competent Ad R700.

To test the importance of capsid proteins on the synergism of adenoviruses and LPS, Ad R700 virions were inactivated with heat or proteinase K treatment (as specified in *Materials and Methods*). The results obtained with these virus preparations are shown in Fig. 4*B*. Unlike UV inactivation, heat and proteinase K treatment totally diminished the activity of adenoviruses. It seems that virions with intact capsid proteins are essential, but de novo viral gene expression is not necessary to achieve synergism with LPS.

Different adenovirus serotypes augment LPS-induced TNF production, but empty capsids are inactive

To test whether adenoviruses belonging to three different subgenera elicit similar responses, Ad12 and Ad3 preparations were used along with Ad R700. In the case of Ad R700 and Ad3, "empty" capsid preparations were also purified to treat B6 mice as described in *Materials and Methods*. All different serotypes and their UV-inactivated preparations increased LPS-induced TNF levels dramatically (Fig. 5). Surprisingly, animals injected with empty capsid preparations failed to show up-regulated TNF production after LPS challenge, suggesting again that intact virions are required for LPS sensitization.

Detection of other cytokines in adenovirus-infected LPS-challenged animals

To study whether the endotoxin-induced production of other acute phase cytokines was also altered in adenovirus-infected mice, serum levels of IL-6 and IFN-γ were determined from selected samples taken 2, 4, and 8 h after the LPS challenge of UV R700-infected mice. Serum TNF values with enhanced LPS-triggered TNF production similar to what was demonstrated earlier are shown on Fig. 6*A*.

In the serum of UV Ad R700-infected mice, no detectable IL-6 level was found. Uninfected, endotoxin-treated animals had their peak serum levels of IL-6 at the 2-h time point. These values

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FIGURE 5. Different Ad serotypes synergize with LPS. Empty capsids are inactive. B6 mice (5/group) were uninfected (8) or infected with 3×10^8 PFU Ad R700 (1 and 9), or with Ad12 (2 and 10) or with Ad3 (3 and 11). The same quantity of UV-inactivated preparations of Ad R700 (4) and Ad 3 (5) and with empty capsids of Ad R700 (6) and Ad3 (7)—normalized for equal protein content with respect to the replication-competent virus—were used. The animals were injected with 1 μg/g LPS (1–8) or with PBS (9–11). Sera were taken 2 h after endotoxin treatment and analyzed for TNF with bioassay. Data are presented as the mean ± SEM.

FIGURE 6. The effect of adenovirus infection on the level of various cytokines after LPS challenge. Serum samples of uninfected (solid bars) and UV R700 (equivalent to 3×10^8 PFU R700) treated (empty bars) B6 mice (5/group) challenged with 1 μg/g body weight LPS were taken 2, 4, and 8 h after LPS challenge and assayed for TNF (*A*), IL-6 (*B*), IFN-γ (*C*), and NO (*D*). Data are presented as the mean ± SEM.

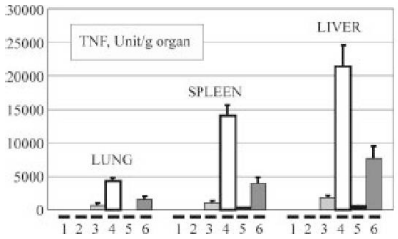

FIGURE 7. TNF levels in different organs of infected mice after LPS challenge. B6 mice (5/group) were infected with UV R700 (equivalent to 3×10^8 PFU Ad R700) (2, 4, and 6) or left uninfected (1, 3, and 5) and treated with 1 μg/g LPS (2–6). Liver, spleen, and lung samples were taken 2 (3 and 4) or 4 (5 and 6) h after endotoxin challenge, homogenized, and cell-associated TNF levels were measured with bioassay. Bars 1 and 2 represent animals without endotoxin treatment. TNF values are shown in U/g organ weight. Data are presented as the mean ± SEM.

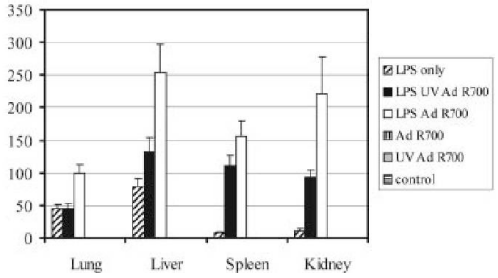

FIGURE 8. NO production in different organs of adenovirus-infected mice after LPS challenge. NO production in different organs of the animals was measured by electron spin resonance as described in *Materials and Methods*. The animals were treated with LPS alone (1 μg/g) or with LPS 16 h after UV-irradiated or non-irradiated adenovirus (R700) infection. No NO was detected in animals treated with adenoviruses alone (irradiated or non-irradiated) or in control animals.

decreased but were readily detectable at the 4-h time point, and at 8 h after LPS challenge, IL-6 levels dropped to near baseline. Both the kinetics and the magnitude of IL-6 production were significantly altered in virus-infected animals (Fig. 6*B*). IL-6 values peaked at 4 h after LPS infusion, and the difference between uninfected and infected animals was maximal (8- to 9-fold) at this time point.

IFN-γ levels were also measured from these serum samples (Fig. 6*C*). Virus infection alone did not produce a detectable induction of the cytokine but resulted in a 2- to 4-fold increase of IFN-γ levels at the later (4 and 8 h) time points in LPS-treated animals. Thus, in the case of this cytokine the difference between uninfected and infected animals was much less pronounced than with TNF or IL-6. From these samples the levels of the IL-10 were also measured (Fig. 6*D*.). LPS treatment resulted in the slight induction of this cytokine.

Increased TNF production in different organs of adenovirus-infected animals upon LPS challenge

To evaluate the effect of adenovirus infection on tissue TNF levels of LPS-challenged mice, liver, spleen, and lung samples from UV-irradiated R700 virus-infected animals were homogenized and analyzed for TNF by bioassay (serum TNF values of the same animals were shown on Fig. 6*A*). The results are shown on Fig. 7. We found that TNF levels in the organs of adenovirus plus LPS-treated mice were much higher than those of the animals treated with LPS alone. TNF levels were the highest in the liver and spleen, which are the main targets of adenoviruses after systemic Ad infection. Surprisingly, in the lung, which harbors only minute parts of the virus under the described conditions, virus infection still had a very significant (5- to 6-fold) effect on LPS-induced TNF levels.

TNF levels decreased much slower in the organs than in the serum (Figs. 1 and 6). Because serum TNF levels increase more drastically in adenovirus-infected animals than organ TNF levels, it seems that serum and tissue concentrations of TNF are differently targeted by adenovirus infection.

Production of NO is greatly enhanced in adenovirus and LPS-treated animals

NO is an important mediator of endotoxin shock and was shown to be induced by TNF. Therefore, we analyzed tissue NO levels in adenovirus-infected LPS-treated mice.

In mice treated with LPS alone, NO was trapped in the liver whereas it was barely detectable in the spleen and kidney. This observation is in agreement with recent studies on LPS-treated mice (22). Adenovirus infection itself did not produce detectable levels of NO in any of the organs. However, in adenovirus-infected LPS-challenged mice the tissue-specific pattern of the detected NO levels was altered dramatically. In the spleen and kidney, where NO was barely detected after LPS treatment alone, pre-infection with Ad R700 or UV-irradiated Ad R700 led to the production of this inflammatory mediator at high levels (Fig. 8).

The effect of virus infection and LPS treatment on the mRNA levels of various cytokines

B6 mice were infected with Ad R700 and UV-inactivated Ad R700 and mRNA samples were purified from the animals 2 and 6 h after LPS induction. Cytokine gene expression was measured by RNase protection assays using a probe set detecting multiple cytokine mRNAs. The levels of different cytokine mRNAs were quantified using a PhosphorImager and the ImageQuant program. Values were normalized to the intensity of the signal of the housekeeping gene, *L-32*. As shown on Fig. 9, replication-competent or UV-inactivated adenovirus infection induced an increase of TNF, IL-6, IL-1α, and IL-1β mRNA levels after LPS challenge in all organs tested.

In different organs, relative ratios of the induced cytokine mRNAs were different, e.g., in the kidney, IL-6 was more effectively induced than IL-1α and IL-1β, whereas the situation was the opposite in the liver. Certain cytokine mRNAs such as IL-2, IL-3, IL-4, and IL-5 were not detectable in any of the samples. Kidney and lung—organs that are not targeted by adenovirus after systemic infections—showed interesting differences. TNF mRNA levels were highly increased in the kidney of mice but in the lung there was only a slight increase in TNF mRNA levels at the 2-h time point after LPS infusion (Fig. 9, *C* and *D*). IL-6 mRNA levels were induced 3- to 4-fold in all four organs tested at 2 h, but only kidney and lung proved to be positive at the 6-h time point. Induction of IFN-γ was demonstrated in spleen and liver at the 6-h time point.

Worth mentioning is the large difference between the degrees of induction of TNF protein and mRNA (which was corroborated by Northern blot analysis, data not shown). Adenovirus infection increased TNF mRNA levels in LPS-treated animals only 3- to 4-fold in the kidney and liver, less in the spleen and lung. The effect of virus infection on LPS-induced TNF protein levels in the same organs were 5–10 times higher (Fig. 7) than the increases in mRNA levels. This suggests that adenovirus-triggered enhancement of LPS-induced TNF synthesis also involves posttranscriptional regulatory steps.

Activation of TLR4 is responsible for LPS-induced cytokine expression (23). TLR4 has been shown recently to mediate responses to respiratory syncytial virus and mRNA expression of

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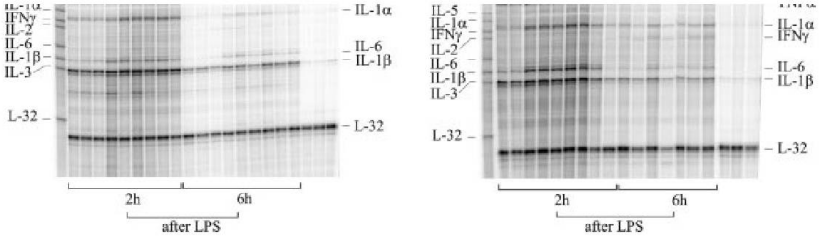
FIGURE 9. The effect of adenovirus infection on the mRNA levels of various cytokines in different organs after LPS challenge. RNA samples from liver, spleen, kidney, and lung of uninfected, Ad R700 and UV Ad R700 (3×10^8 PFU or its equivalent) infected B6 mice were analyzed with RNase protection assay using the ML-11 probe set (*A–E*). Animals were treated or not treated with 1 μg/g LPS, and tissue samples were taken at the indicated time periods after endotoxin challenge as indicated; 0 represents uninfected LPS-treated animals, and C (control) mice received neither LPS nor virus. For the assays, the undigested ML-11 probe set was run at the leftmost lane of each gel as m.w. marker. Data are presented as the mean ± SEM.



TLR1, -2, -3, and -7 was up-regulated in influenza A and Sendai virus-infected macrophages (24, 25). Therefore, we studied the expression of TLR4 in adenovirus-infected animals. We measured the levels of TLR4 and β-actin mRNAs in liver, spleen, kidney, and lung samples of adenovirus and mock-infected mice with quantitative real-time RT-PCR. These results showed that the levels of TLR4 mRNA did not change significantly upon adenovirus infection (data not shown).

Special features of adenovirus-LPS synergism in TNF induction

Similar sensitization to LPS was described earlier in the case of several viruses (26–28). The importance of T cells and IFNs, mainly IFN-γ, was proved in these experiments. We conducted experiments to understand the importance of T cells and IFN-γ in the synergism of adenoviruses and endotoxin in TNF induction.

First, CD1 nu/nu mice lacking mature T cells and immunocompetent CD1 control animals were used. The data obtained using CD1 nu/nu mice are shown on Fig. 10*A*. Adenovirus infection and LPS treatment synergized as effectively in the nude animals as in the control CD1 strain as shown in the previous experiments. The importance of IFN-γ was investigated in IFN-γ $-/-$ mice. As shown on Fig. 10*B*, the total lack of the cytokine had no effect on the phenomenon. UV Ad R700 infection resulted in >100-fold induction of endotoxin-triggered TNF production in the knockout animals. In conclusion, unlike in experiments with other viruses, T cells and IFN-γ do not seem to play any significant role in adenovirus sensitization to LPS.

Because Kupffer cells are a major source of TNF in LPS-treated animals, we performed experiments to assay their role in adenovirus-triggered sensitization to endotoxin. We blocked the function of Kupffer cells with gadolinium treatment (19) and compared TNF induction in treated and control animals. Blockade of Kupffer cells with $GdCl_3$ decreased but did not eliminate the synergism of adenovirus with LPS (Fig. 10*C*). Because rapid production of TNF after LPS challenge is a clear indication of the involvement of the

FIGURE 10. Efficient Ad-endotoxin synergism in mice deficient for T cells, IFN-γ, and Kupffer cells. T cell-deficient, IFN-γ knockout, or Kupffer cell depleted mice (5/group) were infected with UV-inactivated Ad R700 (equivalent to 3×10^8 PFU) or left uninfected. The animals were challenged 16 h later with 1 μg/g LPS. Sera were taken 2 h after endotoxin treatment and analyzed for TNF with bioassay. *A*, CD1 and CD1 nu/nu mice were used to evaluate the effect of T cell deficiency (1 and 3, uninfected; 2 and 4, UV R700 treated). *B*, B6 mice, genetically deficient for IFN-γ uninfected (1) and UV R700 treated (2). *C*, B6 mice, uninfected (1 and 2) or UV R700 infected (3 and 4), after $GdCl_3$ treatment to block Kupffer cells (2 and 4) or without $GdCl_3$ treatment (1 and 3).

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FIGURE 11. Adenovirus infection augments LPS-induced TNF production in human PBMC in vitro. PBMCs were uninfected or treated with UV R700 at a multiplicity of infection of 100. The cells were treated with LPS or left non-stimulated 16 h after infection. TNF was measured from the cell-free supernatant of the cultures with ELISA. Pentoxifylline was used in three different concentrations to inhibit TNF production. P, PBMC; V, virus (UV Ad R700); L, LPS; F, pentoxifylline. V + L represents ELISA background control using medium without PBMC but with virus and LPS. Data are presented as the mean ± SEM.

monocyte-macrophage compartment (containing large pools of preformed TNF mRNA), this experiment suggests that Kupffer cells are important participants in the observed phenomenon.

Adenovirus infection up-regulates endotoxin-induced TNF production in human cells in vitro

Adenovirus infection also increased LPS-induced TNF production in human PBMCs. UV-inactivated Ad R700 was used, and endotoxin stimulation was as described in *Materials and Methods*. The results are shown on Fig. 11. Adenovirus infection alone triggered a slight TNF synthesis similar to data published recently (29). When virus-infected PBMCs were stimulated with LPS they produced 12-fold more TNF than cells treated with endotoxin alone. Pentoxyfylline, a known inhibitor of TNF synthesis was also used in this system and decreased TNF production of adenovirus/LPS-treated PBMCs in a dose-dependent manner.

Adenovirus infection increases lethality after LPS challenge

TNF is a crucial factor in endotoxin-induced death (30), and we have shown the overproduction of this factor in adenovirus- and LPS-treated mice. Therefore, we evaluated the effect of adenovirus infection on endotoxin-induced lethality of experimental animals. Neither the injection of 5 μg/g LPS nor infection with Ad R700, UV-inactivated Ad R700 or Ad3 alone resulted in death of the animals (Fig. 12). However, infection with any one of these viruses sensitized the mice to subsequent endotoxin challenge significantly. Both Ad R700 and UV Ad R700 infection led to 70–80% mortality by the third day after LPS treatment, whereas Ad3-infected mice showed an even higher sensitivity; 100% of the animals died by 2 days after endotoxin challenge. Body temperature of the animals was monitored, and the adenovirus plus LPS-treated animals were rather hypothermic, especially before they died (data not shown). This was found to be characteristic for endotoxin shock in rodents (31).

FIGURE 12. Adenovirus infection exaggerate LPS toxicity. B6 mice (7–10/group) were uninfected (*A*) or infected with Ad R700 (*B*), UV Ad R700 (*C*), or Ad3 (*D*) with 3×10^8 PFU virus or equivalent. The animals were treated with 5 μg/g body weight LPS (■) or PBS (□) 16 h after infection and monitored for survival.

Discussion

Similar to infection with some other viruses, adenovirus exposure increased the sensitivity of experimental animals or isolated human PBMCs to LPS. Expression of adenoviral genes were not needed to elicit this synergism; like wild-type virus, adenovirus mutant dl312, lacking the transactivator gene *E1A* (and consequently expressing other viral genes at very low level), or UV-inactivated adenovirus preparations dramatically increased TNF induction. In contrast, intact virion structure was necessary as heat-inactivated virus preparations or empty virus capsids failed to synergize with LPS. Empty adenovirus particles enter the host cell but do not reach the nucleus (32). Thus, the effect of adenoviruses that confers hypersensitivity to LPS is confined to the part of viral entry that lies between viral endocytosis and nuclear entry. The time interval between viral infection and LPS administration proved to be important. LPS treatment had to follow adenovirus infection by ~16 h to achieve optimal synergism. LPS sensitization seemed to be a general characteristic of adenoviruses, because different adenoviruses belonging to three different subgenera elicited very similar responses. Because adenovirus serotype 3, which is coxsackie virus-adenovirus receptor-independent, was equally capable of eliciting synergism with LPS, binding to coxsackie virus-adenovirus receptor is not specifically required to augment the endotoxin response. Importantly, the synergism of adenoviruses with LPS was not restricted to rodent systems. Adenovirus infection of human PBMCs up-regulated the LPS-induced TNF production of these cells significantly.

It is remarkable that adenovirus infection affected various LPS-induced mediators differently. Among proinflammatory cytokines, the production of TNF was dramatically increased by adenovirus infection, and the synthesis of IL-6 and IFN-γ was also up-regulated. In contrast, the production of IL-10, an anti-inflammatory cytokine was only slightly altered. Most significantly, the LPS-induced synthesis of NO was considerably altered in that it was produced at high levels in organs where endotoxin treatment alone did not induce significant NO production. The overproduction of NO probably played an important role in the augmented lethality

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in that NO was shown previously to be a key mediator in septic shock. To our knowledge, this is the first time that overproduction of this important mediator has been demonstrated in an LPS hypersensitivity model.

Earlier studies described the effect of adenovirus infection on various immune functions. Ginsberg et al. (33) have shown that inhalation of replication-competent adenoviruses caused pneumonia with a local inflammatory reaction characterized by the presence of polymorphonuclear leukocytes and the induction of TNF, IL-1, and IL-6. Early viral gene expression was needed to achieve these events.

Infecting mice i.v. with large amounts (10^{10} PFU) of defective adenoviruses, it was demonstrated that the infection induced cytokines. *E1* deleted but not "gutless" (*E1*, *E2*, *E3*, and late genes deleted) adenovirus vectors triggered the elevation of serum TNF and IL-6, the former in a totally Kupffer cell-dependent manner (34). We used a much lower (10^{8-9} PFU) dose that by itself did not elicit increased serum or tissue cytokine levels. Moreover, Kupffer cells did not seem to play a crucial role in LPS sensitization.

The influence of infection with adenovirus vectors on LPS-induced lung inflammation was studied (35) in an in vivo mouse model measuring TNF and IL-6 in the bronchoalveolar lavage fluid. They found no synergism between adenovirus and LPS treatment. The discrepancy between these findings and our data can be explained by two factors. First, the targeted organs are different. Second, we found that adenovirus infection must precede LPS infusion with at least 7–8 h for sufficient synergism, whereas in the studies of Thorne et al. (35), adenovirus infection was concomitant with LPS treatment.

Most recently, Yarovinsky et al. (36) have shown that adenovirus infection of mice resulted in decreased mortality from LPS and D-galactosamine-induced fulminant hepatitis. The system used in this study differs from ours, because they used D-galactosamine sensitization with a low dose (0.05 μg/g of body weight) of LPS and a longer 7-day adenovirus infection period before the LPS challenge. In contrast, we used 50 μg/g LPS 16 h after adenovirus infection and no sensitization to liver cell apoptosis. Under these circumstances our results clearly showed that adenovirus infection can sensitize to endotoxin shock.

Sensitization to LPS by different pathogens was studied in vivo and in vitro. Endotoxin hypersensitivity in mice was demonstrated with prior treatment with bacteria such as *Propionibacterium acnes* or *Salmonella typhimurium* (37). In mice infected asymptomatically with lymphocytic choriomeningitis virus, LPS induced the superproduction of TNF, IL-12, and IFN-γ (38). Lethality was accelerated by low doses of LPS. IFN-γ seemed to play a crucial role in the augmented TNF production, because TNF levels were much attenuated in experiments using IFN-γ knockout mice (39).

It was also shown that HIV-infected individuals reacted with an elevated production of TNF, IL-6, and IL-8 to LPS challenge and that HIV-infected macrophages produced increased amounts of TNF, IL-1β, and IL-6 after endotoxin treatment; although infected cells alone did not produce these cytokines (40). In macrophage cultures, influenza A and Sendai viruses were shown to activate proinflammatory cytokines including IL-1β, IL-6, IL-18, IFN-α, IFN-β, and TNF at late stages of virus replication and synergism with LPS was demonstrated (41, 42).

An important characteristic of all the above in vivo experiments was the requirement of several days to achieve LPS hypersensitivity that was then sustained for a relatively long time. In all the examples involving viruses, there was a need for replication-competent viruses. In contrast, adenovirus-induced sensitization to LPS developed in 12–16 h but did not last longer than 2 days, and adenovirus replication or viral gene expression was not necessary. Another unique characteristic of the system described here was the altered induction kinetics of the inflammatory cytokines.

The effect of adenovirus infection does not appear to be the overall enhancement of the pathways triggered by endotoxin, but it rather affects mechanisms not targeted by LPS alone. We think that these mechanisms are also different from the ones responsible for the earlier described induction of TNF production by adenovirus alone, for the following reasons: 1) regarding synergism with LPS, treatment with adenovirus showed saturation at a virus titer that, by itself, was not enough to elicit increased TNF production, 2) viral gene expression was not required to achieve adenovirus-LPS synergism, and 3) adenovirus-LPS synergism was not totally Kupffer cell dependent.

An important feature of the adenovirus-LPS synergism was the requirement for a sufficiently long time period between adenovirus infection and the endotoxin challenge. This implies that there was a need for a time-consuming signaling event or for a protein to be synthesized de novo. Because we have shown that viral gene expression was not involved in the augmentation of TNF synthesis if such a protein is produced, then it should be a host protein. In mice infected systemically with adenovirus, the main targeted organs are the liver and spleen, although there are only minute amounts of the input virus in the kidney and lungs. Nevertheless, we detected increased LPS-triggered TNF generation in each of these organs, suggesting the existence of a putative adenovirus-induced soluble factor that could in turn modify LPS responsiveness in uninfected cells.

Adenoviral entry has been recently shown to activate the cAMP-dependent protein kinase A and p38/MAPK signaling pathways (43) and the transcription factor NF-κB (34). These events may contribute to the production of the proposed putative soluble factor that modifies the LPS response.

The effect of adenovirus infection seems to affect multiple levels of regulation because elevation of TNF mRNA levels was observed in the liver and kidney of adenovirus-LPS challenged mice, but TNF protein levels were increased in all studied organs. Furthermore, protein induction was many times higher than the increase in mRNA levels.

A number of other clinical and experimental studies have demonstrated that secondary bacterial infections can significantly worsen the outcome of different primary viral infections. Notably, significant synergism between Ad serotype 1 and a Gram-negative bacterium, *Haemophilus influenzae* was observed in a chinchilla model of experimental otitis media (44). There are various other clinical situations when adenovirus sensitization to LPS may occur. As pulmonary viral infections are often complicated with bacterial ones, it is quite possible that concurrent infections contribute to the altered cytokine levels in the patients. Mixed adenovirus and *H. influenzae* infection, associated with a worse outcome of the disease has also been demonstrated in children (45). A recently described adenovirus-related fatal complication of bowel transplantation was characterized by septic shock-like symptoms and disseminated adenovirus disease (46).

Recombinant adenovirus vectors are promising candidates for various kinds of gene therapy in humans (47–51). To achieve a high frequency of transduction the required virus amounts are large in most of the protocols, so the phenomenon described here may represent a potential complication in the use of recombinant adenoviruses. Indeed, in a fatal adenovirus gene therapy experiment, the patient died due to a disseminated inflammatory reaction (51).

In summary, the data presented here describe and characterize a so far unknown effect of adenovirus infection. They provide more

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insights into the adenovirus-induced innate immune responses emphasizing the importance of TNF and help to understand certain complications of adenovirus-related diseases. The results also highlight the significance of concomitant infections in the hyperstimulation of the immune system.

Disclosures

The authors have no financial conflict of interest.

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